Formation of 17-Allylamino-Demethoxygeldanamycin (17-AAG) Hydroquinone by NAD(P)H:Quinone Oxidoreductase 1: Role of 17-AAG Hydroquinone in Heat Shock Protein 90 Inhibition

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Abstract

We have examined the role of NAD(P)H:quinone oxidoreductase 1 (NQO1) in the bioreductive metabolism of 17-allylamino-demethoxygeldanamycin (17-AAG). High-performance liquid chromatography (HPLC) analysis of the metabolism of 17-AAG by recombinant human NQO1 revealed the formation of a more polar metabolite 17-AAGH₂. The formation of 17-AAGH₂ was NQO1 dependent, and its formation could be inhibited by the addition of 5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxymethyl]indole-4,7-dione (ES936), a mechanism-based (suicide) inhibitor of NQO1. The reduction of 17-AAG to nitrophenoxy)methyl]indole-4,7-dione (ES936), a mechanism-based inhibitor of NQO1. The reduction of 17-AAG to the corresponding hydroquinone 17-AAGH₂ was confirmed by tandem liquid chromatography-mass spectrometry. 17-AAGH₂ was relatively stable and only slowly underwent autooxidation back to 17-AAG over a period of hours. To examine the role of NQO1 in 17-AAG metabolism in cells, we used an isogenic pair of human breast cancer cell lines differing only in NQO1 levels. MDA468 cells lack NQO1 due to a genetic polymorphism, and MDA468/NQ16 cells are a stably transfected clone that express high levels of NQO1 protein. HPLC analysis of 17-AAG metabolism using cell sonicates and intact cells showed that 17-AAGH₂ was formed by MDA468/NQ16 cells, and formation of 17-AAGH₂ could be inhibited by ES936. No 17-AAGH₂ was detected in sonicates or intact MDA468 cells. Following a 4-hour treatment with 17-AAG, the MDA468/NQ16 cells were 12-fold more sensitive to growth inhibition compared with MDA468 cells. More importantly, the increased sensitivity of MDA468/NQ16 cells to 17-AAG could be abolished if the cells were pretreated with ES936. Cellular markers of heat shock protein (Hsp) 90 inhibition, Hsp70 induction, and Raf-1 degradation were measured by immunoblot analysis. Marked Hsp70 induction and Raf-1 degradation was observed in MDA468/NQ16 cells but not in MDA468 cells. Similarly, downstream Raf-1 signaling molecules mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase and ERK also showed decreased levels of phosphorylation in MDA468/NQ16 cells but not in MDA468 cells. The ability of 17-AAG and 17-AAGH₂ to inhibit purified yeast and human Hsp90 ATPase activity was examined. Maximal 17-AAG–induced ATPase inhibition was observed in the presence of NQO1 and could be abrogated by ES936, showing that 17-AAGH₂ was a more potent Hsp90 inhibitor compared with 17-AAG. Molecular modeling studies also showed that due to increased hydrogen bonding between the hydroquinone and the Hsp90 protein, 17-AAGH₂ was bound more tightly to the ATP-binding site in both yeast and human Hsp90 models. In conclusion, these studies have shown that reduction of 17-AAG by NQO1 generates 17-AAGH₂, a relatively stable hydroquinone that exhibits superior Hsp90 inhibition.

Introduction

Heat shock protein (Hsp) 90 has been developed as a potential anticancer target and is an attractive target for several reasons (1–3). Hsp90 is a protein chaperone that uses the hydrolysis of ATP to assist in the folding of early nascent forms of client proteins to their mature, correctly folded forms. Once the client protein has been correctly folded, Hsp90 is released, and as such, it functions as a true protein “catalyst.” The basis for Hsp90 as an anticancer target is that this chaperone assists in the folding of many oncogenic proteins. Such proteins include ErbB2, Raf-1, mutant p53, estrogen, and steroid receptors. Thus, by inhibiting Hsp90, one can target a large number of downstream proteins and thereby attack the neoplastic process at several points (2–4). The first Hsp90 inhibitor used clinically was geldanamycin, which did not move forward in clinical trials due to liver toxicity. Second-generation derivatives, such as 17-allylamino-demethoxygeldanamycin (17-AAG) and 17-dimethylaminogeldanamycin (17-DMAG), do not induce liver toxicity, have completed phase I, and are currently entering phase II clinical trials (2, 3, 5, 6). The product of cytochrome P450–mediated dealkylation of 17-AAG and 17-DMAG at the 17 position, 17-AG, retains its quinone functionality and is also a Hsp90 inhibitor (7).

Because 17-AAG and related benzoquinone ansamycins contain a quinone moiety, bioreduction to semiquinone and hydroquinone species is a possible metabolic pathway within tumor cells, and formation of these species will depend on the levels of bioreductive enzymes. Among the bioreductive enzymes expressed in cancer cells poised to have the greatest influence on 17-AAG metabolism is NAD(P)H:quinone oxidoreductase 1 (NQO1; DT-diaphorase, EC 1.6.99.2). This flavoenzyme can use either NADH or NADPH as reducing cofactors and can catalyze the direct two-electron reduction of quinones to hydroquinones (8). NQO1 is expressed at high levels in many human cancers, including lung, colon, stomach, pancreatic, and breast (9–11). NQO1 has also been shown to

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increase the cytotoxicity of many quinone-containing antimtumor drugs, such as AZQ, mitomycin C, E09, streptonigrin, RH-1, and β-lapachone, by reduction to hydroquinone species (12–17). Clinical trials that target tumors expressing high levels of NQO1 with quinone-based agents are currently in progress.

The role of NQO1 in benzoquinone ansamycin metabolism was first described by Kelland et al. (18). In this work, the authors showed a positive correlation between 17-AAG sensitivity and NQO1 expression. 17-AAG was shown to undergo NQO1-mediated reduction using purified NQO1, and human cancer cell lines expressing NQO1 were more sensitive to 17-AAG (18). We have extended these studies to examine the properties of the hydroquinone formed after reduction of 17-AAG by purified NQO1. We also used the human breast cancer cell line MDA468 (NQO1 null) and an isogenic paired cell line MDA468/NQ16, differing only in NQO1 used the human breast cancer cell line MDA468 (NQO1 null) and an isogenic paired cell line MDA468/NQ16, differing only in NQO1 expression. 17-AAG (5-methoxy-1,2-dimethyl-3-[(4-nitrophenox y)methyl]indole-4,7-dione (ES936)) to confirm the role of NQO1 in the bioreduction of 17-AAG. Studies with purified yeast and human Hsp90 showed that 17-AAGH₂ was a more potent inhibitor of Hsp90, and molecular modeling studies confirmed that 17-AAGH₂ had a greater affinity for the ATP-binding site in yeast and human Hsp90. Our studies show that 17-AAG can be reduced to 17-AAGH₂ by NQO1 and that the hydroquinone is a more potent Hsp90 inhibitor than 17-AAG.

Materials and Methods

Materials. 17-allylamino-demethoxygeldanamycin (17-AAG) was provided by the National Cancer Institute and Kohan Biosciences (Hayward, CA). 2,6-Dichlorophenol-indophenol (DCPIP), NADH, NADPH, sodium borohydride, 3-(4,5-diethyliothiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin (BSA), β-lapachone, N-phényl-1-naphthylamine, n (-)-pinellamine, and mouse monoclonal (IgG) and anti-actin antibodies were obtained from the Sigma Chemical Co. (St. Louis MO). Yeast Hsp90 and radicicol were obtained from Alexxis (San Diego, CA). Human Hsp90 was a kind gift from Dr. David Toft (Mayo Clinic College of Medicine, Rochester, MN). Recombinant human NQO1 (rhNQO1) was purified from Escherichia coli as described previously (19). The activity of rhNQO1 was 4.5 μmol/DCIP/min/mg protein. Malachite green phosphate assay kit was obtained from BioAssay Systems, Inc. (Hayward, CA). Mouse anti-Hsp70 and rabbit anti-Raf-1 antibodies were obtained from Stressgen (Vancouver, British Columbia, Canada). Rabbit anti–mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK)/phospho-MEK and EBK/phospho-ERK antibodies were obtained from Cell Signaling (Beverly, MA).

Cell lines. The human breast cancer cell line MDA468 and the NQO1 stably transfected cell line MDA468/NQ16 have been described previously (20). Cells were grown in RPMI 1640 containing 10% (v/v) fetal bovine serum and 1% (v/v) penicillin, streptomycin, and glutamine. Sonicates were separated by 12% SDS-PAGE (precast minigel, Bio-Rad, Hercules CA) and then scraped in 200 μL ice-cold 50 mmol/L Tris-HCl (pH 7.4) containing 250 mmol/L sucrose and 5 μmol/L fliavin adenine dinucleotide. Protein concentrations were determined by the method of Lowry et al. (21).

Inhibition of NAD(P)H:quinone oxidoreductase 1 by 5-methoxy-1,2-dimethyl-3-[(4-nitrophenox y)methyl]indole-4,7-dione. ES936 was synthesized by Christopher J. Moody (Department of Chemistry, University of Exeter, Exeter, United Kingdom). ES936 has been shown previously to be a potent mechanism-based inhibitor of NQO1 (22). For these studies, a single dose of 100 μmol/L ES936 was nontoxic and resulted in >96% inhibition of NQO1 activity after 4 hours in MDA468/NQ16 cells. Inhibition of rhNQO1 by ES936 was >98%.

High-performance liquid chromatography analysis. The metabolism of 17-AAG by NQO1 was analyzed by high-performance liquid chromatography (HPLC) on a Luna C₁₈ reverse-phase column (5 μm, 4.6 × 250 mm, Phenomenex, Torrance, CA) at room temperature. HPLC conditions were as follows: buffer A, 50 mmol/L ammonium acetate (pH 4) containing 10 μmol/L d(-)-penicillamine; buffer B, acetoni trile (100%). Both buffers were continuously bubbled with N₂ gradient, 20% B to 85% B over 20 minutes and then 85% for 4 minutes (flow rate of 1 mL/min). The sample injection volume was 50 μL. LC-mass spectrometry (MS) was done using positive ion electrospray ionization and the mass spectra were obtained with a PE Scxie API-3000 triple quadrupole MS (Foster City, CA) with a turbo ion spray source interfaced to a PE Scxie 200 HPLC system. Samples were chromatographed on a Luna C₁₈ reverse-phase column (5 μm, 50 × 2 mm, Phenomenex) using a gradient elution consisting of a 2-minute initial hold at 20% B followed by an increase to 80% B over 20 minutes at a flow rate of 0.2 mL/min and a sample injection volume of 20 μL. HPLC conditions were as follows: buffer A, 10 mmol/L ammonium acetate containing 0.1% (v/v) acetic acid (pH 4.4); buffer B, 10 mmol/L ammonium acetate in acetoni trile containing 0.1% (v/v) acetic acid. The MS settings were turbo ion spray temperature of 250°C, spray needle voltage at 4,500 V, declustering potential at 35 V, and focus plate at 125 V. Mass spectra were continuously recorded from 150 to 1,000 amu every 3 seconds during the chromatographic analysis.

Growth inhibition assays. Growth inhibition was measured using the MTT assay. Cells were seeded at 2 × 10⁵ cells per well (96-well plate) in complete medium overnight. The next morning, the cells were pretreated with 100 mmol/L ES936 or an equal amount of DMSO for 30 minutes and then exposed to 17-AAG for 4 hours, after which cells were rinsed free of drug and incubated in fresh medium for an additional 72 hours. Cell viability was measured by the MTT assay as described previously (20).

17-Allylamino-demethoxygeldanamycin metabolism in MDA468 and MDA468/NQ16 cells. The metabolism of 17-AAG in cells was done under the following conditions: Cells were seeded at 2 × 10⁶ cells per 100-mm plate (in duplicate) in complete medium for 3 days. Cells were then pretreated with 100 mmol/L ES936 or an equal amount of DMSO for 30 minutes followed by 5 μmol/L 17-AAG for additional 4 hours at 37°C. Following drug treatment, cells (one plate) were washed extensively in PBS containing 1% (v/v) BSA and then lysed on the plate by the addition of 200 μL ice-cold acetoni trile containing 1 μg/mL N-phényl-1-naphthylamine (internal standard). Samples were centrifuged at 13,000 rpm for 10 seconds and the supernatant was immediately analyzed by HPLC. The cell number was determined using the second plate of cells. Cells were washed in PBS and then scraped in 200 μL PBS and counted (hemocytometer).

Immunoblot analysis. MDA468 and MDA468/NQ16 cells were grown in 100-mm plates in complete medium to ~70% confluency. For Hsp70 and Raf-1 analysis, cells were treated with DMSO or 17-AAG (0.5-1 μmol/L) in 10 mL complete medium for 8 hours. For MEK and ERK analysis, cells were serum starved for 24 hours and then treated with DMSO or 17-AAG (0.5-1 μmol/L) in serum-free medium for 8 hours. Following drug treatment, cells were washed in PBS and then lysed by the addition of radio-immunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 0.5% (v/v) NP40] containing 1 Mini protease tablet (protease inhibitor cocktail, Roche, Indianapolis, IN) and phosphatase inhibitors (30 μmol/L NaF, 40 μmol/L β-glycerophosphate, 20 mmol/L sodium pyrophosphate, 1 mmol/L orthovanadate, 1 mmol/L EGTA). Lysates were probe sonicated (2 seconds) on ice and then centrifuged to remove cellular debris. Protein concentrations were determined on supernatant by the method of Lowry et al. (21). Samples were heated to 70°C in 2× Laemmli SDS sample buffer, and proteins were separated by 12% SDS-PAGE (precast minigel, Bio-Rad, Hercules CA) and then transferred to 0.4-μm polyvinylidene difluoride membranes. Membranes were blocked in 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.2% Tween 20, and 5% nonfat milk for a minimum of 1 hour at room temperature. Anti-Hsp70, anti-Raf-1, anti-MEK, and anti-ERK antibodies were added for 1 hour at room temperature. Anti- phospho-MEK and ERK antibodies were added overnight at 4°C. All primary antibodies were diluted 1:1,000 except actin (1:5,000). Horseradish peroxidase–labeled secondary antibodies (Jackson ImmunoResearch Labs, West Grove, PA) were diluted 1:5,000 and added for 30 minutes. Proteins were visualized using enhanced chemiluminescence detection.

Heat shock protein 90 ATPase activity assay. Inhibition of Hsp90 ATPase was measured as described previously (24). Briefly, purified yeast or
human Hsp90 (2.5 μg) was incubated in 100 mmol/L Tris-HCl (pH 7.4) containing 20 mmol/L KCl, 6 mmol/L MgCl₂, 400 μmol/L NADH, 17-AAG with or without 3.3 μg rhNQO1, and 20 μmol/L ES936. Reactions (25 μL) were started by the addition of 1 mmol/L ATP and allowed to proceed at 37°C for the indicated times. Reactions were then diluted with 225 μL of 100 mmol/L Tris-HCl (pH 7.4) containing 20 mmol/L KCl and 6 mmol/L MgCl₂ mixed thoroughly, and 80 μL were transferred to each well (96-well plate) followed by 20 μL malachite green reagent. After 10 minutes, trisodium citrate (83 mmol/L) was added to stabilize the color and plates were read at 650 nm.

Molecular modeling of 17-allylamo-demethoxygeldanamycin and 17-AAGH₂ in yeast and human heat shock protein 90 crystal structures. Molecular modeling studies were done on a Silicon Graphics Octane2 workstation. The crystallographic coordinates for the 2.5-Å structure of yeast Hsp90 (PDB: 1A4H; ref. 25) and the 1.9-Å structure of human Hsp90 (PDB: 1YET; ref. 26) were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank.¹ The Hsp90 crystal structures were visualized using the InsightII software package version 2000 (Accelrys, Inc., San Diego, CA). The Builder Module was used to add hydrogens to the protein structure and the ionizable Asp, Arg, Glu, and Lys residues were corrected for physiologic pH. The 17-AAG and the corresponding hydroquinone, 17-AAGH₂, structures were constructed and assigned the correct atom type and bond order, from the bound geldanamycin structure, in each Hsp90 crystal structure. Once constructed, the ligands were in turn superpositioned onto the bound geldanamycin structure using the coordinated system of the protein to correctly position the ligand in the geldanamycin-binding domain of Hsp90. The geldanamycin scaffold structure was then deleted from the protein structure and the “docked” ligand assembly was associated with the Hsp90 protein structure. For the molecular mechanics and molecular dynamics calculations, the Discover Module was used and the potentials and charges of the Hsp90-ligand complex were corrected using the consistent-valence force field. The Hsp90-ligand complex was then minimized using the conjugate gradient method (1,000 iterations). The Docking Module was used to perform the intermolecular energy calculation to determine the nonbonded interaction energy between Hsp90 and the appropriate ligand. An interface 6-Å radius subset encompassing the ligand-binding domain was selected and both van der Waals and electrostatic (coulombic) energies were calculated with a specified cutoff of 8 Å.

¹ http://www.rcsb.org

Figure 1. HPLC and LC-MS analysis of the reduction of 17-AAG by NQO1. HPLC analysis confirmed the formation of 17-AAGH₂ following reduction of 17-AAG by rhNQO1 (A and B) and inhibition of this reduction by ES936 (C). Reaction conditions: 50 μmol/L 17-AAG, 200 μmol/L NADH, and 3.3 μg rhNQO1 in 50 mmol/L potassium phosphate buffer (pH 7.4; 1 mL) containing 1 mg/mL BSA. After 40 minutes, reactions were stopped with an equal volume of acetonitrile containing internal standard N-phenyl-1-naphthylamine (5 μg/mL) and centrifuged and the supernatant was analyzed immediately by HPLC at 334 nm. A, 17-AAG and NADH; B, 17-AAG, NADH, and rhNQO1; C, 17-AAG, NADH, rhNQO1, and ES936 (1 μmol/L). The relatively small peak size of 17-AAGH₂ compared with 17-AAG is due to low absorption at 334 nm for the hydroquinone. Inset, NQO1-mediated metabolism of 17-AAG (same conditions as in B) at a detection wavelength of 270 nm where the 17-AAG quinone and hydroquinone have approximately equal absorption. LC-MS was used to confirm 17-AAGH₂ as the product of NQO1-mediated reduction of 17-AAG (D).
Results

In vitro studies with purified rhNQO1 clearly showed that reduction of 17-AAG by NQO1 in combination with NADH or NADPH generated 17-AAGH\textsubscript{2}. HPLC analysis of aerobic incubations of 17-AAG, NADH, and rhNQO1 resulted in loss of the 17-AAG peak and generation of the more polar metabolite 17-AAGH\textsubscript{2} (Fig. 1). Formation of 17-AAGH\textsubscript{2} was NQO1 dependent and could be inhibited by addition of the NQO1 mechanism-based inhibitor ES936. Identification of the more polar product as 17-AAGH\textsubscript{2} was confirmed by LC-MS (Fig. 1D). 17-AAGH\textsubscript{2} formed following reduction of 17-AAG by NQO1 and NADH coeluted with the major product generated during sodium borohydride reduction of 17-AAG (data not shown). HPLC analysis showed that 90% of 17-AAG was detected in the form of the hydroquinone after reduction by NQO1, suggesting the generation of a relatively stable hydroquinone. During analysis, HPLC buffers were continuously gassed with N\textsubscript{2}, and a copper chelator (see Materials and Methods) was included. Preliminary data (not included) showed that inclusion of copper (CuSO\textsubscript{4}) could facilitate 17-AAGH\textsubscript{2} oxidation. The stability of 17-AAGH\textsubscript{2} in solution was measured by HPLC following reduction of 17-AAG by rhNQO1 using stoichiometric equivalents of NADH and 17-AAG (Fig. 2). In these studies, the autooxidation of 17-AAGH\textsubscript{2} was followed over time in an aerobic reaction. These results showed that 17-AAGH\textsubscript{2} undergoes a slow rate of autooxidation back to 17-AAG over a period of hours.

Whether 17-AAGH\textsubscript{2} could be formed in cells following reduction by NQO1 was investigated using the isogenic human breast cancer cell lines MDA468 and MDA468/NQ16. These cell lines have been used previously to examine the role of NQO1 in antitumor quinone metabolism (20). The parental MDA468 cell lines is NQO1 null (<10 nmol DCPIP/min/mg) due to homozygous expression of the NQO1*2 polymorphism. The stable transfection of the MDA468 cell line with human NQO1 generated the MDA468/NQ16 cell line, which has high NQO1 activity (>1,800 nmol DCPIP/min/mg). Initial experiments were done using cell sonicates prepared from MDA468 and MDA468/NQ16 cells. HPLC analysis of these reactions was nearly identical to results obtained with rhNQO1 (Fig. 1A). Sonicates prepared from MDA468/NQ16 cells readily generated 17-AAGH\textsubscript{2} (Fig. 3A). Formation of 17-AAGH\textsubscript{2} was NADH or NADPH-dependent and could be inhibited by addition of ES936 (Fig. 3B). HPLC analysis of 17-AAGH\textsubscript{2} formation by MDA468 and MDA468/NQ16 cell sonicates showed that 90% of 17-AAG was detected in the form of the hydroquinone after reduction by NQO1, suggesting the generation of a relatively stable hydroquinone.
dependent and could be inhibited by ES936 (Fig. 3B). No 17-AAGH2 could be detected in sonicates from MDA468 cells. To further investigate the role of NQO1 in the reduction of 17-AAG, we examined whether 17-AAGH2 could be generated by NQO1 in intact cells. In these experiments, MDA468 and MDA468/NQ16 cells were treated with 17-AAG (5 μmol/L) for 4 hours and then washed free of drug and lysed by the addition of acetonitrile containing internal standard. Concentrations of 17-AAG and 17-AAGH2 were then measured by HPLC, and the results clearly showed that 17-AAGH2 could be generated in MDA468/NQ16 cells and formation of 17-AAGH2 could be inhibited by pretreatment with ES936 (Fig. 3C). No 17-AAGH2 could be detected in MDA468 cells. In addition to generating 17-AAGH2, MDA468/NQ16 cells also accumulated more total drug (quinone plus hydroquinone) compared with cells treated with ES936 or parental MDA468 cells (Fig. 3C). Cell viability was >90% for both cell lines under these treatment condition (trypan blue exclusion assay).

The role of NQO1 in growth inhibition induced by 17-AAG was measured in MDA468 and MDA468/NQ16 cells. In these studies, cells were treated with 17-AAG for 4 hours in the presence and absence of ES936 pretreatment. Cells were then washed free of drugs and growth inhibition was determined using the MTT assay (Fig. 4). Results from these experiments showed that MDA468/NQ16 cells had increased sensitivity to 17-AAG (IC50 0.86 ± 0.16 μmol/L) compared with parental MDA468 cells (IC50 10.05 ± 1.07 μmol/L). The sensitivity to 17-AAG could be abrogated by pretreatment with ES936 (IC50 7.67 ± 1.36 μmol/L). Results were similar using an 8-hour exposure to 17-AAG with an ~20-fold increase in sensitivity in MDA468/NQ16 cells relative to MDA468 cells, and growth inhibition in MDA468/NQ16 cells could also be abrogated by ES936. Experiments were also done using a nonquinone Hsp90 inhibitor, radicicol, as a negative control. The growth-inhibitory effects of radicicol were essentially identical in MDA468 and MDA468/NQ16 cells (data not shown), showing the requirement for a quinone functionality for increased growth inhibition in MDA468/NQ16 cells.

The role of NQO1 in 17-AAG-induced Hsp90 inhibition in MDA468 and MDA468/NQ16 cells was also examined. To measure Hsp90 inhibition in these cells, we analyzed Hsp70 induction and Raf-1 degradation as markers of Hsp90 inhibition (Fig. 4). These studies showed greater Hsp70 induction and increased Raf-1 degradation in MDA468/NQ16 cells compared with MDA468 cells. We then examined downstream (Raf-1) signaling as reflected in MEK and ERK phosphorylation in MDA468 and MDA468/NQ16 cells.
cells treated with 17-AAG (Fig. 4). Decreased levels of both MEK and ERK phosphorylation were observed in MDA468/NQ16 cells compared with MDA468 cells following serum starvation. No changes in total MEK or ERK protein levels were observed (Fig. 4). These data suggested that a greater level of Hsp90 inhibition was occurring in NQO1-rich MDA468/NQ16 cells compared with NQO1-deficient MDA468 cells.

To confirm the relationship between 17-AAGH₂ formation and increased Hsp90 inhibition, we examined the ability of 17-AAG and 17-AAGH₂ to inhibit the ATPase activity of purified yeast and human Hsp90 (Fig. 5). In these assays, we incubated purified yeast or human Hsp90 with 17-AAG (2 μmol/L) and NADH (400 μmol/L) in the presence and absence of rhNQO1. Reactions were allowed to proceed for 3 hours (yeast Hsp90) or 12 hours (human Hsp90), after which reactions were terminated and the concentration of inorganic phosphate was measured using the malachite green assay (24). Results from experiments using yeast Hsp90 showed that 17-AAGH₂ was the active Hsp90 inhibitor. In these studies, a substantial decrease in ATPase activity was observed with 17-AAG in the presence of NQO1 and this could be prevented by ES936. No inhibition by 17-AAG was observed in the absence of NQO1. Results from experiments using human Hsp90 were qualitatively similar but showed some quantitative differences. In these experiments, the addition of 17-AAG resulted in some inhibition of Hsp90 ATPase activity. Inhibition was significantly increased, however, by the inclusion of NQO1 and abrogated by inactivation of NQO1 by ES936 (Fig. 5).

Molecular modeling studies of 17-AAG and 17-AAGH₂ docked into the ATP-binding site of the yeast or human Hsp90 crystal structure revealed significant differences in the binding energies between the two compounds after minimization. The nonbonded interaction energy or binding energy is the sum of the van der Waals and electrostatic energies, the measure of the affinity between Hsp90 and 17-AAG/17-AAGH₂. In both yeast (Table 1) and human (Table 2) Hsp90 crystal structures, 17-AAGH₂ had greater nonbonded interaction energy than the parent quinone. These data support the hypothesis that the hydroquinone form of 17-AAGH₂ is a more potent inhibitor of Hsp90. Following minimization, the Hsp90-ligand complex was visualized to identify important amino acid residues in the ATP-binding domain that interact via hydrogen bonding with the ligand investigated (Fig. 6); there was no significant change in the global conformation of Hsp90. However, the Hsp90-17-AAGH₂ complex revealed additional hydrogen bonding interactions between the hydroquinone moiety and Hsp90 that resulted in a greater binding energy (Tables 1 and 2).

In the ATP-binding domain of yeast Hsp90, the C21 ketone of 17-AAG hydrogen bonds with the amine of Lys₁₀⁸ and the C18 ketone of the quinone ring system interacts with a water molecule that in turn contacts Asp₅⁴, whereas in 17-AAGH₂ the oxygen atom of the C21 hydroxyl does not seem to interact directly with Lys₁₀⁸. The hydrogen atom of the C18 hydroxyl interacts with an oxygen atom of the carboxylate side chain of Asp₁⁴², which subtly alters the overall conformation of 17-AAGH₂ and allows the amide of the ansa ring to interact with the backbone nitrogen of Phe₁³⁶. Interestingly, no interactions were observed with yeast Hsp90 and the 17-(2-propenylamino)-substituent; the side chain is orientated into solvent and does not interfere with the binding of the ligand to the target site.

Similar interactions were observed in the ATP-binding domain of human Hsp90 with 17-AAG; the C21 ketone hydrogen bonds with the amine of Lys₁¹² and the C18 ketone interacts with a water molecule that makes contact with Asn⁵¹. In both 17-AAG and 17-AAGH₂, the amide of the ansa ring interacts with the backbone nitrogen of Phe₁³⁸ and the C11 hydroxyl group hydrogen bonds with the amine of Lys⁸⁶ in human Hsp90. However, there is a rearrangement of protein-ligand interactions in the binding site accompanying the substitution of the hydroquinone for the benzoquinone moiety. The interaction of the C21 hydroxyl and the amine of the Lys₁¹² is maintained, although at a greater hydrogen bond distance, in contrast to that observed with yeast Hsp90. The C18 hydroxyl interacts with Asp₅⁴; as a result, the hydroquinone moiety of 17-AAGH₂ adopts a more compact conformation around the amino acid residues of helix 2. This allows hydrogen bonding between the 17-NH group of the 17-(2-propenylamino)-substituent and the carboxylate side chain of Asp₅⁴ and the side chain amine of Lys⁸⁶. The hydrogen bonding between the 17-amino group of 17-AAGH₂ and Asp₅⁴ and Lys⁸⁶ is the only protein-ligand interaction provided by the 2-propenylamino group, because the remainder of the side chain is orientated away from the binding site and into the solvent.
Discussion

Kelland et al. (18) in the first study of the role of NQO1 in the mechanism of action of benzoquinone ansamycins showed a relationship between levels of NQO1 and sensitivity to 17-AAG. Kelland et al. also reported that NQO1 may play a role in the cellular metabolism and activity of Hsp90 inhibitors because they observed increased 17-AAG and geldanamycin activity in NQO1-transfected BE cells relative to NQO1-null BE parental cells (18). This was an intriguing result and we examined the activity of 17-AAG in the MDA468 and MDA468/NQ16 isogenic cell pair. Consistent with the data of Kelland et al. (18), we found markedly increased 17-AAG growth inhibition in MDA468/NQ16 cells with

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<th>( E_{elect} ) (kcal/mol)</th>
<th>( E_{total} ) (kcal/mol)</th>
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<td>–68.2</td>
<td>Asp(^{72}): hydroquinone O-H 2.40 Asp(^{72}): carbamate NH(_2) 2.00 Phe(^{123}): amide (ansa) C=O 2.35 HOH: carbamate C=O 2.05 HOH: carbamate NH(_2) 2.25 HOH: methoxy (ansa) OCH(_3) 2.30 HOH: carbamate OCONH(_2) 2.50 HOH: hydroxy (ansa) OH 2.38 HOH: hydroquinone O-H 2.33</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: \( E_{total} \), total interaction energy; \( E_{vdw} \), van der Waals; \( E_{elect} \), electrostatic energy.

Table 2. Total interaction energy, van der Waals, electrostatic energy, and hydrogen bonding interactions between human Hsp90 and 17-AAG/17-AAGH\(_2\)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>( E_{vdw} ) (kcal/mol)</th>
<th>( E_{elect} ) (kcal/mol)</th>
<th>( E_{total} ) (kcal/mol)</th>
<th>H-bond Interactions</th>
<th>H-bond Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-AAG</td>
<td>–23.5</td>
<td>–4.6</td>
<td>–28.1</td>
<td>Lys(^{98}): hydroxy (ansa) O-H 1.69 Asp(^{54}): amine NH 2.27 Lys(^{98}): hydroxy (ansa) O-H 1.72 Lys(^{98}): amine NH 2.44 Asp(^{54}): carbamate NH(_2) 2.04 Lys(^{112}): hydroquinone O-H 1.90 Phe(^{138}): amide (ansa) C=O 2.00 HOH: carbamate NH(_2) 2.27 HOH: carbamate C=O 2.05 HOH: carbamate OCONH(_2) 2.47 HOH: methoxy (ansa) OCH(_3) 2.32</td>
<td></td>
</tr>
<tr>
<td>17-AAGH(_2)</td>
<td>–22.3</td>
<td>–15.2</td>
<td>–37.5</td>
<td>Asp(^{54}): hydroquinone O-H 1.96 Asp(^{54}): amine NH 2.27 Lys(^{98}): hydroxy (ansa) O-H 1.72 Lys(^{98}): amine NH 2.44 Asp(^{54}): carbamate NH(_2) 2.04 Lys(^{112}): hydroquinone O-H 1.90 Phe(^{138}): amide (ansa) C=O 2.00 HOH: carbamate NH(_2) 2.27 HOH: carbamate C=O 2.05 HOH: carbamate OCONH(_2) 2.47 HOH: methoxy (ansa) OCH(_3) 2.32</td>
<td></td>
</tr>
</tbody>
</table>
high NQO1 relative to the NQO1-deficient parental MDA468 cell line. The MDA468 cell line is deficient in NQO1 because the homozygous NQO1*2 polymorphism that we have shown previously leads to rapid degradation of NQO1*2 protein (27). Radicicol, a non-quinone-containing Hsp90 inhibitor, showed essentially identical growth-inhibitory profiles in MDA468 and MDA468/NQ16 cells showing the requirement for a quinone functionality for increased sensitivity in cells containing elevated NQO1 levels. The interesting aspect of our data was that we were able to extend previous work by use of ES936, a mechanism-based inhibitor of NQO1 (22). We showed that inhibition of NQO1 with ES936 could abrogate the growth-inhibitory activity of 17-AAG in NQ16 cells, raising IC50 values back to approximately those observed in the parental MDA468 cells. Increased client protein inhibition and downstream Raf-1 mediated signaling could be shown in NQ16 cells relative to MDA468 cells. These observations argued strongly that 17-AAGH2 was a more potent inhibitor than 17-AAG itself.

Using HPLC analysis, we showed the generation of 17-AAG hydroquinone from 17-AAG, by recombinant NQO1, in NQ16 sonicates and intact cells but not in MDA468 sonicates or cells. The generation of 17-AAGH2 by recombinant NQO1 was verified by LC-MS. The production of the hydroquinone of 17-AAG in NQ16 sonicates and cells could be blocked by use of ES936, the suicide inhibitor of NQO1, which was entirely consistent with cellular growth inhibition and client protein inhibition data. In agreement with previous data (28), it seems that aside from leading to the formation of 17-AAGH2 the presence of NQO1 in a cell also leads to

Figure 6. Molecular modeling of the Hsp90-17-AAG/17-AAGH2 complex. Flat ribbon representation of the yeast Hsp90 ATP-binding domain with (A) 17-AAG and (B) 17-AAGH2 and human Hsp90 ATP-binding domain with (C) 17-AAG and (D) 17-AAGH2 (ligands are yellow; stick display style) displaying hydrogen bond contacts (green dashed lines) with key amino acid residues and water molecules (stick display style). The carbon atoms are in gray, hydrogen atoms are in white, nitrogen atoms are in blue, and oxygen atoms are in red unless otherwise stated. The figures were constructed using Discovery Studio Viewer Professional Software (Accelrys).
increased total accumulation of (quinone plus hydroquinone) ansamycin equivalents. Presumably, this reflects the increased water solubility of 17-AAGH₂ and a decreased propensity of the hydroquinone once generated in a cellular system to cross membranes. Thus, reduction of 17-AAG by NQO1 may not only lead to the generation of the more potent form of the Hsp90 inhibitor but also result in the retention of the more active hydroquinone form in the cell (Scheme 1).

To confirm our hypothesis that the hydroquinone was the more potent Hsp90 inhibitor, we proceeded with two lines of experiments. In the first, we used purified yeast and human Hsp90 and employed established methods to assess the ATPase activity of these purified proteins. We showed that in studies with both yeast and human Hsp90 the generation of 17-AAGH₂ by NQO1 was required for maximal ATPase inhibition. The inhibition of ATPase activity could be abrogated by use of ES936, the mechanism-based inhibitor of NQO1, confirming the crucial role of 17-AAGH₂ in Hsp90 inhibition.

The second line of experimentation we pursued was computer modeling of the NH₂-terminal domain of Hsp90 containing the ATPase active site. Although the complete structure of the intact Hsp90 protein has not yet been determined, crystal structures of the NH₂-terminal domain identified by limited proteolysis have been described for yeast (25) and human (26) Hsp90. The tertiary structures of these amino acid terminal domains are very similar, containing a β-sheet covered on one face by α-helices, with 69% identity yeast to human, consistent with the high homology among all Hsp90 sequences. The helical face of the Hsp90 NH₂-terminal domain has a wide opening at its center that extends into the hydrophobic core of the structure and results in a pronounced pocket, the binding site for the benzoquinone ansamycin antagonist agents, geldanamycin and 17-DMAG (25, 26, 29).

The 17-AAG and 17-AAGH₂ structures consist of a 17-(2-propanylamino)-substituted planar benzoquinone or hydroquinone, embedded in a closed ansa ring system (Fig. 6). The ansa ring is folded under the benzoquinone/hydroquinone, forming a C-shaped conformation with the carbamate group at the base of the binding site and the benzoquinone/hydroquinone ring at the solvent exposed pocket entrance. Multiple protein-ligand interactions lock the macrocycle of 17-AAG and 17-AAGH₂ in an overall conformation similar to that of geldanamycin and 17-DMAG (25, 26, 29).

The greater interaction energies of 17-AAGH₂ is explained by the stronger and greater number of hydrogen-bonding interactions with key amino acid residues in the ATP-binding domain of Hsp90. As a result, 17-AAGH₂ adopts a more compact C-shaped, clamp-like conformation around helix 2 due to the hydrogen bonding interactions of the C18 hydroxyl group, of the hydroquinone moiety, with Asp⁴⁰ of yeast Hsp90 and Asp⁵³ of human Hsp90, thereby increasing the electrostatic contribution to the nonbonded interaction energy.

It is clear that NQO1-mediated generation of 17-AAGH₂ from 17-AAG results in increased Hsp90 inhibition as indicated by effects on client proteins and growth inhibition and that NQO1 is a critical determinant of maintaining the benzoquinone ansamycin in its more potent hydroquinone form in tumors. Dependent on their structure and redox potential, however, hydroquinones exhibit varying stability toward oxygen. The propensity for hydroquinones to undergo autooxidation varies and depends on oxygen tension, redox potential, and availability of redox active metals, particularly iron and copper. Very little is known about the stability of 17-AAGH₂ and the hydroquinone forms of other ansamycins. Previous work focusing on geldanamycin reported the preparation of the geldanamycin hydroquinone by sodium dithionite reduction, which was stable enough to be isolated and purified but reverted to the hydroquinone slowly in the presence of air (7). Our data show that the hydroquinone derivative of 17-AAG is relatively stable and in agreement with the previous report (7); we found that the hydroquinone slowly reoxidizes back to the parent quinone on exposure to air over a period of hours. This suggests that the pharmacokinetic and pharmacodynamic studies of the effects of benzoquinone ansamycins need to consider the quinone and hydroquinone as separate entities, particularly as they possess very different abilities to inhibit Hsp90.

Our work on the formation of 17-AAGH₂ by NQO1 was presented in abstract form (30), and at the same meeting, data were shown that proposed the use of the hydroquinone of 17-AAG as a watersoluble prodrug of 17-AAG (31). The development of 17-AAGH₂ as a therapeutic entity makes it even more important to study the biological properties of the benzohydroquinone ansamycins, their stability and their generation in human tumors.

In summary, our data show that 17-AAGH₂ can be generated selectively in tumor cells containing high levels of NQO1 and that the hydroquinone form, based on target inhibition in both cell-free and cellular systems and molecular modeling studies, represents a more potent inhibitor of Hsp90.

**Acknowledgments**

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*Scheme 1. Proposed model of inhibition of cellular Hsp90 by 17-AAG and 17-AAGH₂. Major pathways are shown as solid lines; minor pathways are shown as dotted lines.*
Formation of 17-Allylamino-Demethoxygeldanamycin (17-AAG) Hydroquinone by NAD(P)H:Quinone Oxidoreductase 1: Role of 17-AAG Hydroquinone in Heat Shock Protein 90 Inhibition

Wenchang Guo, Philip Reigan, David Siegel, et al.


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